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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C05F 11/08, C12N 1/04</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/06732</b> <b>(43) International Publication Date:</b> 31 March 1994 (31.03.94)
<b>(21) International Application Number:</b> PCT/BR93/00032 <b>(22) International Filing Date:</b> 10 September 1993 (10.09.93)  <b>(30) Priority data:</b> PI 9103905 11 September 1992 (11.09.92) BR  <b>(71) Applicant (for all designated States except US):</b> GERATEC S/A - BIOTECNOLOGIA APLICADA [BR/BR]; Rodovia Br. no. 158, Km 206, Distrito Industrial, Cruz Alta, RS (BR).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> ABRAÃO, José, Joaquim, Rodrigues [BR/BR]; Rua Barão do Rio Branco no. 1485, Aptº 101, Cruz Alta, RS (BR).  <b>(74) Agent:</b> P.A. PRODUTORES ASSOCIADOS MARCAS E PATENTES LTDA.; Rua Dr. César no. 796, 2º Andar, Santana, 02013-000-São Paulo, SP (BR).		<b>(81) Designated States:</b> AU, CA, JP, NZ, RU, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD FOR OBTAINING A WETTABLE POWDER INOCULANT FOR USE WITH LEGUMINOUS CROPS  <b>(57) Abstract</b>  The invention refers to a method for obtaining a wettable powder inoculant formulation for use with leguminous crops, comprising the industrialization of an inoculant for leguminous from a centrifugated biological mass of Rhizobium spp twice immersed in a cryogenic protective solution rich in sugar and protein which is subjected to a lyophilization method. After a lyophile having a concentration of $1.5 \times 10^{11}$ of viable cells/gram has been obtained, it is formulated with substances rich in polysaccharides and associated with a cellular protector which substitutes the previously used peat support. The new formulation is advantageous when compared with the peat-supported inoculants because it has higher adherence to the seeds, it can be directly applied to the seeds in the sowing box, it does not cause an increased wear of the sowing machines, and it has a storage useful life of up to 25 months even when stored at room temperature.		

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## Specification

"METHOD FOR OBTAINING A WETTABLE POWDER  
INOCULANT FOR USE WITH LEGUMINOUS CROPS"

## Field of the Invention

5           The invention refers to a method for obtaining  
a wettable powder inoculant elaborated from *Rhizobium* spp  
and formulated as a wettable powder for use with leguminous  
crops, whose useful life is increased up to 25 months,  
contrary to other formulations made with peat supports,  
10   which have a useful life of only 6 months. The inoculant  
according to the present invention is also characterized by  
having a greater degree of stickness to the seeds and by  
not requiring storage at low temperatures.

## Background of the Invention

15           The inoculant according to the present  
invention is a biological fertilizer having a peat support  
and comprising more than 50% of organic material, enriched  
with a *Rhizobium* type bacteria.

          The inoculation method according to the present  
20   invention comprises mixing the inoculant with the seeds  
slightly prior to sowing them in order to promote a higher  
degree of symbiosis between the *Rhizobium* spp and the  
seeds, which results in the formation of nodules in the  
roots of the plants through which the nitrogen present in  
25   the atmosphere is absorbed and transferred to the plant,  
therefore partially or completely substituting with  
atmospheric nitrogen the nitrogen-based fertilizers  
presently used with leguminous crops.

          Notwithstanding the positive effect which can

easily be confirmed by the higher productivity of the leguminous crops, the use of an inoculant manufactured with a peat support still find a strong resistance from Brazilian farmers due to the following reasons: its application requires the use of additional people; it increases the wear of the sowing machines; it does not easily adhere to the seeds; it requires special storage and conservation conditions, with temperatures below 15 °C; the increases in productivity can not be easily confirmed; and its storage life of 6 months. On the other hand, industries also find it difficult to use peat due to: the unevenness and low quality of the peat (an acid fibrous peat with a high content of sand and a small capacity of retaining water); the need of extracting great volumes of peat which have to be dried and treated, the treatment usually employing methods that generate extremely unhealthy environmental conditions; the continuous extraction of the peat might cause irreversible damages to the environment; not to mention the above-cited problems related to its formulation and application.

In order to overcome the above-listed disadvantages related with the industrialization and application of inoculants manufactured from peat, researchers have developed other promising techniques for manufacturing the inoculant, among which liophilization, using as support a vegetal or mineral oil, peat, organic waste from crops, vegetal or mineral coal and clays (talcum, bentonite, vermiculite). All the above referred supports, however, suffer from problems related to the

3

application of the product or the survival of the Rhizobium. The following works corroborate the above affirmative: Kremer & Petersen (1983), in the United States, noticed that the Rhizobium spp under a lyophilized form and using an oil or a vegetable coal as support still would have, after 125 days stored at a temperature of 35 °C a survival rate well above the one presented by the Rhizobium spp supported on grounded peat. In another work, Kremer et al (1982) also noticed similar results in an inoculant supported by an oil stored both at 35 °C and 65 °C.

Additionally, a lyophilized inoculant supported by an oil is now being manufactured in Brazil, but with some problems with respect to the stability of the suspension and of the survival of the Rhizobium spp. This is confirmed by Scholles et al (1990), which have analyzed several commercially available Brazilian products comprising lyophilized Rhizobium spp supported in oil, only to notice that from 6 samples none presented the minimum concentration required by Brazilian law.

Notwithstanding the benefits which result from inoculation, it is believed that over 50% of the farmers do not use inoculants because of the poor quality of the inoculants and to the above cited problems. This fact has caused an effort from researchers to provide improved inoculant formulations for use with leguminous crops which reduce to a minimum the problems related to manufacturing, application and agronomical efficiency.

With this purpose in mind, the present

inventors started from a *Rhizobium* spp culture, leavened for 72 hours, centrifugated and lyophilized. Thereafter, the lyophile was formulated as a wettable powder wherein the peat inoculant bath was replaced by a lyophile and the peat replaced by inert supports rich in polysaccharides, associated to a celular protector, which imparts to the new formulation the following characteristics: (1) it easily adhres to the seeds, because it has adhesives in the formulation; (2) it is economic and easy to use because it provides, after it has been jellied in water, a direct mixing with the seeds in the planting box; (3) it can be formulated with inert supports which do not cause any harm to the sowing machines because they do not have any abrading substances in their composition; (4) it has a storage useful life of up to 25 months, therefore well superior to the 6 months useful life of the inoculants supported by peat or peat; (5) it maintained its agronomical efficiency after it had been manufactured and also maintained its concentration of *RHizobium* spp in a level higher than the provided by the Brazilian law, for a period of up to 25 months, which is the greatest advantage provided by the inoculant according to the present invention, mainly because up to now it was not known that any commercially available *Rhizobium* spp product could survive for so long stored at normal conditions.

#### Manufacturing Process

SEMIA 587 and 5019 stocks, usually recommended for soy-bean crops were multiplied for 72 hours in self-sterilizing stainless reactors with a capacity of 1500

5

liters and aerated with 1 V/V up to reaching the stage of logarithmic growth. The media of the culture was then sterilized at 120 °C for about 1 hour and kept at 28 °C with constant agitation at 350 rpm and comprised the

5 following substances:

	Maninol .....	10.0 g/l
	Yeast water .....	100.0 m/l
	Potassium phosphate, dibasic .....	0.5 g/l
	Magnesium sulphate .....	0.2 g/l
10	Ammonium phosphate, monobasic .....	0.5 g/l
	Potassium nitrate .....	1.0 g/l
	Sodium chloride .....	0.2 g/l
	Iron sulphate .....	0.01 g/l
	Manganese sulphate .....	0.01 g/l
15	Water qsq .....	1.000 ml
	pH corrected to .....	7.0

The concentration of the *Rhizobium* spp during the fermentation was evaluated by counting the cells in a Neybauer chamber with a biocular microscope, at 400X, and

20 also by inoculating a Petry's plaque with an agaric culture media and a Congo red marker. After the bath, comprising a mixture of SEMIA 587 and 5019 from *Rhizobium japonicum*, had fermented and reached a logarithmic growth stage with a concentration of  $1.7 \times 10^9$  viable cells/ml of the bath, it

25 was submitted to centrifugation. A tubular centrifuge IMA, model T-7 with a continuous flow and a capacity of 300 l/hour was used in the centrifugation process, where the supernatant was eliminated and the precipitate presented an yield of 8.0 g/l of biological mass with a water content of

75%.

A criogenic protective solution comprising 5.0% of glycerol, 2.0% of gelatin, 2.5% of casein, and 100 ml of de-ionized water qsq, with an adjusted pH of 6.8 and  
5 sterilized at 120 °C for 15 minutes was used to treat the biological mass. The biological mass was twice immersed in the criogenic protective solution with a 50% proportion in order to protect the cells during the method of lyophilization-drying. The drying procedure was conducted  
10 in a FIC lyophilizer model 50-L for 26 hours, and it finally had a concentration of  $1.5 \times 10^{11}$  viable cells/gram of the lyophile with a water content of 1%.

After obtaining the lyophile, a study of several wettable powder formulations using different  
15 special supports with higher adherence to the seeds and higher useful life of the *Rhizobium* spp was conducted. This was determined by using supports rich in sugars, proteins and a celular protector to enhance the rehydration of the bacteria. The following substances can be used in order to  
20 achieve this objective. As celular protectors: saccharose, glucose, casein and peptone. As adhesives: carboxymethylcellulose, powdered arabic gum. As dispersants: tween 85, sodium sulphate lauril. As inerts: caulim, bentonite and silicium bioxide.

25 The amount of lyophile to be used in the formulation depends on the concentration of viable cells. In the formulation of the wettable powder according to the present invention 0.2 g/dose of the lyophile were used, with a final concentration of  $3.0 \times 10^{10}$  viable cells for

each dose of 120 ml of the inoculant. Therefore, each new dose of the inoculant comprises:

	Lyophile ( $1.5 \times 10^{11}$ viable cells/g) .....	0.2 g
	Saccharose (celular protector) .....	0.2 g
5	Sodium sulphate lauril (dispersant) .....	0.5 g
	Kaolin (inert) .....	0.6 g
	Carboxymethyl cellulose (adhesive) .....	1.0 g
	Total .....	2.5 g

Initially, the substances were dried at 105 °C,  
10 untill a constant weight was obtained, and thereafter the formulation was prepared obeying the following steps: (A) the lyophile and the celular protector (saccharose) were mixed together with the dispersant (sodium sulphate lauril); (B) the inert (kaolin) and the adhesive  
15 (carboxymethyl cellulose) were mixed. After the formulation had been balanced, mixtures A and B were mixed, in a V shaped stainless mixer having a capacity of 100 liters mechanically agitated for 20 minutes. The wettable powder formulation was then tightly packaged in 50 ml bottles or  
20 polypropilene bags.

From a point of view of in-site final use, each dose corresponds to 2.5 g of the wettable powder formulation, which is to be diluted in 120 ml of water up to six hours prior to be used. The gelification of the  
25 product in the water forms a gel which easily adhere to the seeds required to cover half hectare. In the case of soy-bean (*Glycine max*), each dose (2.5 g of the formulation + 120 ml of water) shall be applied to 50 Kg of seeds, which amount corresponds to about half hectare.

Agronomical Results Obtained with the Wettable Powder Inoculant Formulation

Three months after the lyophilization process, the analysis of the lyophile showed it contained  $1.5 \times 10^{11}$  viable cells of lyophile/gram (Example 1), which survival rate was maintained for up to 25 months.

From Examples 1 and 4 it can be noticed that the results of infection in plants (NMP), 25 months after the lyophile had been obtained, show a minimum of  $1.7 \times 10^6$  viable cells/gram and that the wettable powder formulation resulting therefrom, initially with  $4.5 \times 10^7$  viable cells/gram, only shows a tendency to reduce the concentration of *Rhizobium* spp after 25 months of storage time at a temperature of 35 °C. It is advisable to remind that the conventional peat-based inoculants have a 6 months storage time and should present at least  $10 \times 10^6$  viable cells/gram six months after it has been manufactured. Therefore, even with 25 months, the wettable-powder formulation according to the present invention is well above the legal provisions about Fertilizers, Correctives and Inoculants.

Example 2 shows the results of experiences conducted at the Green House of the Instituto de pesquisas Agronômicas da Secretaria da Agricultura (IPAGRO-RS). It can be noticed that the results of the formulation of the wettable powder inoculant was superior to that with the same results obtained with a peat-supported inoculant from IPAGRO (official results), in both types of soil, both with respect to the formation of dry nodules as with respect to

the increase of weight of the above ground part of the plant.

In an experience conducted by the Centro de Biotecnologia e Faculdade de Agronomia da UFRGS (Gráfico 1), Silva et al (1992) also noticed increases in over 1.0 t of soy-bean grains, when using the wettable powder inoculant formulation according to the present invention applied through the seeds, when compared to the treatment without the use of the inoculant. Example 3 shows the average results of the grain yield, dry weight of the nodules and the final population of the plants, as well as a comparison of the averages according to Duncan test at 1% and the coefficients of the variations. It can be noticed from comparing the averages that the average grain yield after the treatment with the wettable powder according to the present invention, according to Duncan test at 1% shows that it was statistically similar to that of the peat-based inoculants, but superior to the same result without inoculant.

The analysis of the results of the previously shown experiences conducted allow us to conclude that the concentration of viable cells of the Rhizobium spp in the wettable powder inoculant formulation according to the present invention, 25 months after it was first manufactured, has a concentration above the minimum provided by law, which is  $10 \times 10^6$  viable cells/gram at the last useful day of validity of the product, and this reflected positively in the grain yield of the soy-bean, when compared to the traditionally used peat-supported

inoculants.

#### Advantages

The inoculant according to the present invention formulated as a wettable powder, in addition to not losing its agronomical efficiency, provides a series of advantages when compared with the traditionally used peat-supported inoculants of the prior art, such as:

a) The peat-supported inoculants require the use of additional people, while the wettable powder inoculating formulation according to the present invention does not require the use of additional people because it can be directly applied to the seeds in the planting box.

b) The commercially available inoculants have to be prepared and used when the seeds are being sowed, while the wettable powder inoculating formulation according to the present invention can be prepared up to 6 hours prior to its use.

c) The peats normally used have a high content of sand and silica which increases the wear of the sowing disks in the machines, which does not happen with the wettable powder inoculating formulation according to the present invention which does not have any abrading substances in its formulation.

d) The good adherence of the peat-supported inoculants of the prior art to the seeds depends on the use of adhesives and powder mixing machines, while the wettable powder inoculating formulation according to the present invention easily adheres to the seeds because it has adherents in its composition.

11

e) The commercially available peat-supported inoculants have a useful life of 6 months while the wettable powder inoculating formulation according to the present invention has a useful life of up to 25 months.

# 5 Example 1:

Tests conducted in a Green House by the official method of diluting and infection in plants (NMP) in a lyophilized bath (lyophile) and in the wettable powder inoculating formulation (PM).

10

	Storage Time (Months)	Tests in Plants (NMP)*		
		Lyophile (cells/g)	Wettable Powder Formulation (cells/g/p.c.)	Peat Inoculant
15	3	$1.5 \times 10^{11}$	$4.5 \times 10^7$	$5.8 \times 10^8$
	6	-	$4.5 \times 10^7$	$6.9 \times 10^7$
	6	-	$+1.4 \times 10^7$	$5.9 \times 10^8$
20	12	$1.7 \times 10^{11}$	$1.7 \times 10^{10}$	0**
	15	-	$1.4 \times 10^7$	0
	18	-	$4.5 \times 10^7$	0
	21	$1.5 \times 10^{11}$	$1.7 \times 10^8$	0
	25	$1.5 \times 10^{11}$	$1.7 \times 10^8$	0
25				

\* Average of four repetitions

cells/g/p.c. - amount of viable cells per gram of wettable powder formulation product.

\*\* - Low concentration in which the official method did not detect the presence of Rhizobium spp

30

12

Example 2 - Experiment conducted by IPAGRO-RS, in a green house from UFRGS, with two different types of arenous soil, involving inoculata formulations for soy-bean. The harvesting of the nodules (mg/vase) and of the upper part of the soy-bean (g/vase) was conducted in January/1991 (Jo o Kolling et al, 1991, unpublished).

	Treatments	Dry Weight of Nodules			Plant Weight		
		Soil 1	Soil 2	Aver.	Soil 1	Soil 2	Aver.
10	IPAGRO						
	Inoculant*	131	183	157	2.48	2.94	2.66
	No inoculant	9	9	9	2.10	2.36	2.23
	Moist. powder**						
15	Formulation	147	231	189	2.72	3.05	2.89
	Oil-supported**						
	Formulation	166	110	138	2.66	2.69	2.68

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Soil 1 - Vacacai Soil

20 Soil 2 - São Jerônimo Soil

\* Peat-supported inoculant prepared by IPAGRO

\*\* Wettable powder formulation and oil-supported formulation containing lyophilized *Rhizobium japonicum*

13

Example 3 - Average yield of 5 repetitions of soy-bean grains (Kg/ha), dry weight of the nodules (g), final population of plants/linear meter of variety, Cobb, according to the inoculant used.

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	Treatments	Grain Yield		Dry Weight		Final
		Aver. % No Inoc.	% W/Inoc.	Nodules	Population	
10	No Inoc.	1915 C	100	61.40	0.98 B	20.4 A
	Inoc. <sup>1</sup>	3117 B	162.8	100	2.03 A	21.6 A
	Sterilized Peat-supported					
	Inoc.	3315 BA	173.1	106.40	1.92 A	19.8 A
15	Wettable powder					
	Form.**	3223 BA	168.3	183.40	2.11 A	22.8 A
	Variation	5.835	-	-	10.870	8.815

1- Commercially available peat-supported inoculant

2- Wettable powder formulation

Averages followed by the same letter do not differ from each other according to Duncan test at 1% probability

14

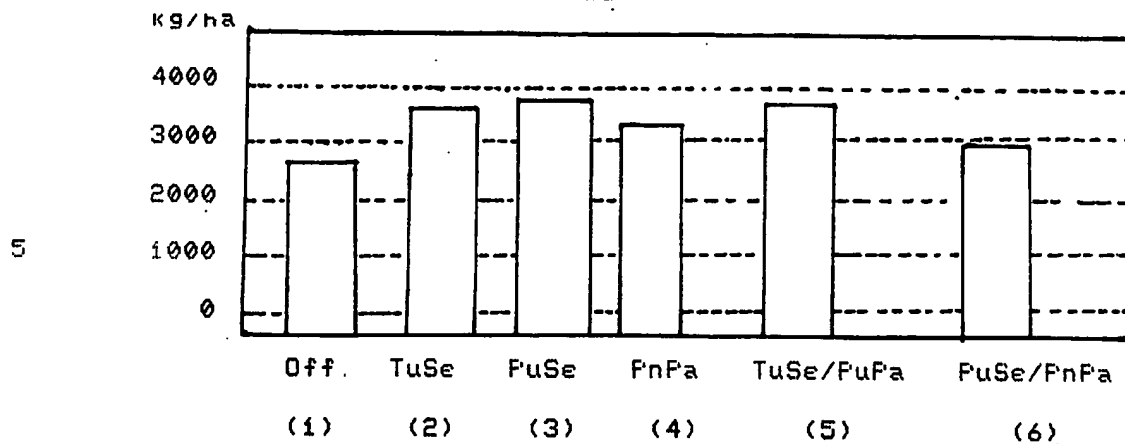
Example 4 - Effect of storage temperature of inoculant with respect to the survival of the Rhizobium spp

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Storage Time		Peat Inoc.*			Moist. Powder Inoc.		
(Months)		15 °C	35 °C	Room	15 °C	35 °C	Room
10	3	$5.8 \times 10^8$	$5.8 \times 10^8$	$5.8 \times 10^8$	$4.5 \times 10^7$	$4.5 \times 10^7$	$4.5 \times 10^7$
	6	$6.9 \times 10^7$	$5.8 \times 10^8$	$6.9 \times 10^7$	$1.7 \times 10^7$	$4.5 \times 10^7$	$4.5 \times 10^7$
	9	$5.9 \times 10^4$	-	$1.8 \times 10^5$	$4.5 \times 10^7$	$1.4 \times 10^7$	$1.4 \times 10^7$
	12	$3.1 \times 10^5$	-	-	$9.3 \times 10^7$	$1.7 \times 10^{10}$	$1.7 \times 10^{11}$
	15	-	-	-	$7.0 \times 10^7$	$1.4 \times 10^7$	$1.4 \times 10^7$
15	18	-	-	-	$4.5 \times 10^7$	$1.8 \times 10^7$	$4.5 \times 10^7$
	21	-	-	-	$1.8 \times 10^7$	$1.7 \times 10^8$	$1.7 \times 10^7$
	25	-	-	-	$4.5 \times 10^7$	$1.7 \times 10^8$	$1.7 \times 10^8$

\* - official method for analysing dilution and infection in plants (NMP): number of viable cells/gram of formulated product

15



10 Graphic 1 - Grain yield in the evaluation experience of the application of the inoculant in covering soy-bean (Silva et al. 1992, M.B., 1992)

(1) - Official Result

(2) - Peat-supported inoculant through the seeds

15 (3) - Wetttable powder inoculant formulation through the seeds

(4) - Wetttable powder inoculant formulation coverage

(5) - Treatment 1 + treatment 2

(6) - Treatment 3 + treatment 4

16  
CLAIMS

1. A method for obtaining a wettable-powder inoculant for use with leguminous crops, characterized by the industrialization, from a biological mass of Rhizobium spp twice immersed in a solution rich in sugars and proteins, comprising casein, glycerol and gelatin, which concentration varies from  $1.5 \times 10^{11}$  to  $1.7 \times 10^{11}$  of viable cells/gram of lyophile, after it has been lyophilized, which is used to substitute the bath for manufacturing the new formulation.

2. A method for obtaining a wettable-powder inoculant for use with leguminous crops, according to claim 1, wherein the peat support is substituted by one of the following supports rich in polysaccharides associated with a celular protector: saccharide, sodium sulphate lauril, kaolin and carboxymethyl cellulose, which impart to the new formulation a higher adherence to the seeds after it has been jellied in water, which formulation can be prepared up to 6 hours prior to its use.

3. A method for obtaining a wettable powder inoculant comprising a lyophile, a celular protector, a dispersant, and adhesive and an inert support for use with leguminous crops, according to claim 1, characterized in that the inoculant has a storage useful life of up to 25 months while still having a minimum concentration  $1.7 \times 10^8$  viable cells/gram of the formulation.

4. A method for obtaining a wettable powder inoculant comprising a lyophile, a celular protector, a dispersant, an adhesive and an inert support for use

17

with leguminous crops, according to claim 1, characterized in that the *Rizobium* spp present in the formulation has a storage useful life of up to 25 months even when stored at room temperature.

## INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/BR 93/00032

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 C05F11/08 C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C05F C12R C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 203 708 (AGRACETUS) 3 December 1986 see claims 1-10 see page 5, line 14 - page 7, line 26 ---	1-4
Y	DE,C,41 09 036 (WABLAT, W.) 13 August 1992 see claims 1-12 see examples 4,7 see page 2, line 36 - line 63 ---	1-4
A	US,A,4 421 544 (JONES, J.L.) 20 December 1983 see claims see column 1, line 56 - column 2, line 37 see column 3, line 28 - line 51 --- -/--	1-4



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

22 November 1993

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# INTERNATIONAL SEARCH REPORT

International Application No  
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DE,A,19 02 118 (SCIENCE-UNION ET CIE., SOCIETE FRANÇAISE DE RECHERCHE MEDICALE) 30 October 1969 see claims 1-5 see page 2, paragraph 2 -----</p>	1-4

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. Appl. Application No

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